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(57) Abstract

The present invention pertains to novel inhibitors of cyclin-dependent kinases (CDKs), particularly CDK/cyclin complexes, which inhibitors can be used to control proliferation and/or differentiation of cells in which the inhibitors are introduced. More specifically, the inhibitors of the invention are chimeric proteins which include CDK-binding motifs from two or more different proteins. For example, the subject chimeric proteins can be generated from the in-frame fusion of coding sequences from two different CDK inhibitor proteins, such as may be derived from fusion of coding sequences for an INK4 protein and coding sequences for a CIP protein. Chimeric proteins of the present invention have been observed to be more potent inhibitors of cyclin/CDK complexes than were either of the portions of the chimeric protein individually.

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INHIBITORS OF CELL-CYCLE PROGRESSION, AND USES RELATED THERETO

Background of the Invention

The cell division cycle is one of the most fundamental processes in biology which, in multicellular organisms, ensures the controlled generation of cells with specialized functions. Under normal growth conditions, cell proliferation is tightly regulated in response to diverse intra- and extracellular signals. This is achieved by a complex network of proto-oncogenes and tumor-suppressor genes that are components of various signal transduction pathways. Activation of a proto-oncogene(s) and/or a loss of a tumor suppressor gene(s) can lead to the unregulated activity of the cell cycle machinery. This, in turn, will lead to unregulated cell proliferation and to the accumulation of genetic errors which ultimately will result in the development of cancer (Pardee, Science 246:603-608, 1989).

In the eukaryotic cell cycle a key role is played by the cyclin-dependent kinases (CDKs). Cdk complexes are formed via the association of a regulatory cyclin subunit and a catalytic kinase subunit. In mammalian cells, the combination of the kinase subunits (such as cdc2, CDK2, CDK4 or CDK6) with a variety of cyclin subunits (such as cyclin A, B1, B2, D1, D2, D3 or E) results in the assembly of functionally distinct kinase complexes. The coordinated activation of these complexes drives the cells through the cell cycle and ensures the fidelity of the process (Draetta, Trends Biochem. Sci. 15:378-382, 1990; Sherr, Cell 73:1059-1065, 1993). Each step in the cell cycle is regulated by a distinct and specific cyclin-dependent kinase. For example, complexes of Cdk4 and D-type cyclins govern the early G1 phase of the cell cycle, while the activity of the CDK2/cyclin E complex is rate limiting for the G1 to S-phase transition. The CDK2/cyclin A kinase is required for the progression through S-phase and the cdc2/cyclin B complex controls the entry into M-phase (Sherr, Cell 73:1059-1065, 1993).

The CDK complex activity is regulated by mechanisms such as stimulatory or inhibitory phosphorylations as well as the synthesis and degradation of the kinase and cyclin subunit themselves. Recently, a link has been established between the regulation of the activity of cyclin-dependent kinases and cancer by the discovery of a group of CDK inhibitors including the $p27^{Kip1}$, $p21^{Waf1/Cip1}$ and $p16^{Ink4/MTS1}$ proteins. The activity of $p21^{Waf1/Cip1}$ is regulated transcriptionally by DNA damage through the induction of p53, senescence and quiescence (Harper et al., Cell 75:805-816, 1993). The inhibitory activity of $p27^{Kip1}$ is induced by the negative growth factor TGF-β and by contact inhibition (Polyak et al., Cell 78:66-

69, 1994). These proteins, when bound to CDK complexes, inhibit their kinase activity, thereby inhibiting progression through the cell cycle. Although their precise mechanism of action is unknown, it is thought that binding of these inhibitors to the CDK/cyclin complex prevents its activation. Alternatively, these inhibitors may interfere with the interaction of the enzyme with its substrates or its cofactors.

While $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ inhibit all the CDK/cyclin complexes tested, $p16^{Ink4/MTS1}$, p15, p18 and p19 block exclusively the activity of the CDK4/cyclin D and CDK6/cyclin D complexes in the early G1 phase (Serrano et al., Nature 366:704-707, 1993), by either preventing the interaction of Cdk4 and Cyclin D1, or indirectly preventing catalysis. As mentioned above, the $p21^{Waf1/Cip1}$ is positively regulated by the tumor suppressor p53 which is mutated in approx. 50% of all human cancers. $p21^{Waf1/Cip1}$ may mediate the tumor suppressor activity of p53 at the level of cyclin-dependent kinase activity. $p16^{Ink4/MTS1}$ is the product of a tumor suppressor gene localized to the 9p21 locus, which is frequently mutated in human cancer cells.

Of all the various kinases, the CDK4/cyclin D complexes are known to play an important role in regulating cell cycle progression in early G1. These complexes function as integrators of various growth factor-induced extracellular signals and as a link between the different signal transduction pathways and other cyclin-dependent kinases. The expression of the cyclin D1 positive regulatory subunit, is deregulated by gene translocations, retroviral insertions and amplifications in parathyroid adenomas, lymphomas, esophageal and breast carcinomas. The targeted overexpression of cyclin D1 in the mammary epithelium of transgenic mice induces mammary adenomas and adenocarcinomas. This confirms that cyclin D1, when overexpressed, acts as an oncogene (Wang et al., Nature 369:669-671, 1994). These data supports the idea that the lack of functional $p16^{Ink4/MTS1}$ or the overexpression of cyclin D1 leads to the deregulation of CDK4/cyclin D1 kinase activity and thereby contribute to uncontrolled cell proliferation.

The prominent role of CDK/cyclin kinase complexes, in particular, CDK4/cyclin D kinase complexes, in the induction of cell proliferation and their deregulation in tumors, makes them ideal targets for developing highly specific anti-proliferative agents.

Summary of the Invention

In one aspect, the present invention relates to a nucleic acid comprising a nucleotide sequence encoding a chimeric polypeptide having at least two CDK-binding motifs derived from different proteins which bind to cyclin dependent kinases (CDKs). The chimeric polypeptide binds to CDKs and inhibits cell-cycle progression.

The chimeric polypeptide can be a fusion protein, or can be generated by chemically cross-linking the CDK-binding motifs.

In preferred embodiments, at least one of the CDK-binding motifs is a CDK-binding motif of a CDK inhibitor protein, such as an INK4 protein, e.g., p15, p16, p18 and p19, or a CIP protein, e.g., $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. However, it will be understood that other CDK-binding motifs may be useful. Indeed, the CDK-binding motif of the INK4 proteins is characteristized by tandemly arranged ankyrin-like sequences, which sequences exist in other proteins and, for those which are able to bind a CDK, can be used to generate the subject chimeric proteins. Likewise, the CDK-binding motif can be a p21/p27 inhibitory domain of a protein which has some homology with the CIP protein family. Exemplary chimeric proteins of the present invention are designated by SEQ ID No. 2, 5 and 7, and are encoded by the CDS's designated in SEQ ID No. 1, 4 and 6.

In preferred embodiments, the CDK-binding motifs of the chimeric protein have different binding specificities, relative to one and other, for cyclin dependent kinases. For instance, the chimeric protein can be generated with a CDK-binding motif from a protein which binds to and inhibits a CDK involved in progression of the cell cycle in G_0 and/or G_1 phase, and another CDK-binding motif from a protein which binds to and inhibits a CDK involved in progression of the cell cycle in S, G_2 and/or M phase. That is, the chimeric protein will bind to and inhibit a plurality (two or more) of cyclin dependent kinases which are active in different phases of the cell-cycle.

In most embodiments, the nucleic acid will further include a transcriptional regulatory sequence for controlling transcription of the nucleotide sequence encoding the chimeric polypeptide, e.g., the transcriptional regulatory sequence is operably linked to a chimeric gene encoding the chimeric polypeptide. For example, the present invention specifically contemplates recombinant transfection systems which include: (i) a gene construct including a nucleic acid encoding a chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases, and operably linked to a

transcriptional regulatory sequence for causing expression of the chimeric polypeptide in eukaryotic cells, and (ii) a gene delivery composition for delivering the gene construct to a cell and causing the cell to be transfected with the gene construct. For example, the gene construct can be derived from a viral vector, such as an adenoviral vector, an adeno-associated viral vector or a retroviral vector. In such embodiments, the gene delivery composition comprises a recombinant viral particle. In other embodiments, the gene construct can be delivered by such means as a liposome or a poly-cationic nucleic acid binding agent. For in vivo delivery to a mammal, such as a human, the gene delivery composition will further include a pharmaceutically acceptable carrier for adminstration to an animal, and, as necessary, will be a sterile preparation and substantially free of pyrogenic agents.

The present invention also pertains to preparations of such chimeric polypeptides. e.g., polypeptides which are generated from CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases. In preferred embodiments, the chimeric polypeptide is formulated in pharmaceutically acceptable carrier for delivery to a mammal. For example, the chimeric polypeptide can be formulated in liposomal preparations.

Still another aspect of the present invention related to transgenic animals which have cells harboring a nucleic acid one of the subject fusion proteins.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C.

Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Detailed Description of the Invention

Progression of eukaryotic cells through the cell cycle is governed by the sequential formation, activation, and subsequent inactivation of a series of cyclin/cyclin dependent kinase complexes. The mechanisms underlying the expression of cyclins and the activation of the different cyclin-CDK complexes needed for progression through successive cell cycle transitions are now fairly well understood. In addition to positive regulation by the activation of cyclin-CDK complexes, negative regulation of the cell cycle occurs at checkpoints, many of which operate to control formation of cyclin/CDK complexes and/or activation of the complexes. Accordingly, these transitions are negatively regulated by signals that constrain the cell-cycle until specific conditions are fulfilled. Entry in to mitosis, for example, is inhibited by incompletely replicated DNA or DNA damage. These restriction on cell-cycle progression are essential for preserving the fidelity of the genetic information during cell division. The transition from G_1 to S phase, on the other hand, coordinates cell proliferation with environmental cues, after which the checks on the cell-cycle progression tend to be cell autonomous. Disruption of these signaling pathways can uncouple cellular responses from environmental controls and may lead to unrestrained cell proliferation or abherrent loss of differentiation.

The present invention pertains to novel inhibitors of cyclin-dependent kinases (CDKs), particularly CDK/cyclin complexes, which inhibitors can be used to control proliferation and/or differentiation of cells in which the inhibitors are introduced. More specifically, the inhibitors of the invention are chimeric proteins which include CDK-binding motifs from two or more different proteins. For example, as set forth in greater detail below, the subject chimeric proteins can be generated from the in-frame fusion of coding sequences from two different CDK inhibitor proteins (generically refered to herein as "CKI" proteins), such as may be derived from fusion of coding sequences for an INK4 protein and coding sequences for a CIP protein. Moreover, as the appended examples describe, chimeric proteins of the present invention have been observed to be more potent inhibitors of cyclin/CDK complexes than were either of the portions of the chimeric protein individually. For instance, p27-p16 chimeric proteins inhibited a cyclin D1/CDK4 complex with an IC_{50} more than two-fold less than p27 alone, and ten-fold less than

p16 alone. Likewise, the p27-p16 chimeric protein inhibited cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDK2 complexes with IC_{50}'s approximately two-fold less than p27 alone (p16 itself not having any significant inhibitory activity against any of the three complexes).

Other aspects of the present invention include: preparations of the subject chimeric proteins; expression constructs for recombinant production of the subject chimeric proteins, particularly for use as part of a gene therapy treatment; and methods for modulating cell proliferation and/or differentiation with the subject chimeric proteins.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The phrase "CDK-binding motif" refers to that portion of a protein which interacts either directly or indirectly with a cyclin dependent kinase (CDK). The binding motif may be a sequential portion of the protein, i.e., a contiguous sequence of amino acids, or it may be conformational, i.e. a combination of non-contiguous sequences of amino acids which, when the protein is in its native folding state, forms a structure which interacts with a CDK. The term "CDK-binding motif" explicitly includes any polypeptide which is identical, substantially homologous, or otherwise functionally or structurally equivalent to a portion of a CKI protein which binds directly or indirectly to a CDK or CDK complex. Other exemplary CDK-binding motifs can be provided from, for example, Rb and Rb-like proteins as well as cyclins.

An "inhibitor of CDK activation" refers to a molecule able to interact with a CDK and prevent activation of a kinase activity of the CDK either by, for example, inhibiting formation of CDK complexes including regulatory subunits, inhibiting interaction of the CDK subunit with activating kinases or phosphatases, inhibiting substrate binding, inhibiting ATP binding, and/or inhibiting conformational changes required for enzymatic activity. Accordingly, such inhibition may be by a direct, competitive mechanism, or by an indirect, non- or uncompetitive mechanism.

As used herein, the term "CKI protein" refers to a protein which can bind to and inhibit activation of a cyclin dependent kinase. Exemplary CKI proteins include members of the INK4 family, such as $p16^{INK4A}$ or $p15^{INK4B}$, and members of the CIP family, such as $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$.

The term "INK4 protein" refers to a family of structurally related CDK inhibitors characterized by a fourfold repeated ankyrin-like sequence (Elledge et al.

(1994) Curr. Opin. Cell Biol. 6:874-878), and the ability to bind to CDKs, especially CDK4 and CDK6. Exemplary members of this protein family include p16 (INK4A/MTS1; Serrano et al (1993) Nature 366:704-707); p15 (INK4B; Hannon et al. (1994) Nature 371:257-261); p18 (Guan et al. (1994) Genes Dev. 8:2939-2952) and p19 (Chan et al. (1995) Mol. Cell Biol. 15:2682-2688; and Hirai et al. (1995) Mol. Cell Biol. 15:2672-2681). Other proteins have been identified in the art as having tandemly arranged ankyrin-like sequences, such as the Pho81p protein (Ogawa et al. (1995) Mol. Cell Biol. 15:997-1004), and may provide CDK-binding motifs which are functionally equivalent to those of an INK4 protein.

The term "CIP protein" refers to members of another CKI protein family which includes $p21^{CIP1}$ (WAF1/SDI1/CAP20; Xiong et al. (1993) Nature 366:701-704); $p27^{KIP1}$ (Polyak et al. (1994) Cell 78:67-74); and $p57^{KIP2}$ (Lee et al. (1995) Genes Dev. 9:639-649; and Matsuoka et al. (1995) Genes Dev. 9:650-662). In addition to the functional characteristic of CDK inhibition, the CIP proteins each have a CDK inhibitory motif (a CDK-binding motif) of about 50 amino acids, referred to herein as a "p21/p27" inhibitory domain, which is conserved in members of the CIP family, as well as, for example, members of the Rb-like protein family.

A "chimeric protein" refers to a protein which includes polypeptide sequences from at least two different and distinct proteins. A chimeric protein can be a fusion protein, or the different polypeptide sequences can be covalently linked by a non-peptide bond, e.g., a cross-linking agent.

As used herein, the term "fusion protein" is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

The art term "fusion gene" refers to a nucleic acid in which two or more genes are fused resulting in a single open reading frame for coding two or more proteins that as a result of this fusion are joined by one or more peptide bonds.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a fusion polypeptide of the

present invention, including both exonic and (optionally) intronic sequences. An exemplary recombinant gene encoding a subject fusion protein is represented by SEQ. ID No: 1.

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein with respect to transfected nucleic acid, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a CDK-inhibitory fusion polypeptide of the present invention.

"Expression vector" refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a DNA sequence encoding a desired protein (in this case, a fusion protein of the present invention) which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. In preferred

embodiments, transcription of the fusion gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of one of the naturally-occurring forms of a CDK inhibitor protein.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a urogenital origin, e.g. renal cells, or cells of a neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

"Recombinant host cells" refers to cells which have been transformed or transfected with vectors constructed using recombinant DNA techniques. As relevant to the present invention, recombinant host cells are those which produce CDK inhibitor fusion proteins by virtue of having been transformed with expression vectors encoding these proteins.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, a bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of a CDK inhibitory fusion protein. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found,

or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that the recombinant gene is present and/or expressed in some tissues but not others.

"Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences.

One aspect of the invention pertains to a nucleic acid having a nucleotide sequence encoding a chimeric CDK inhibitor protein, and/or equivalents of such nucleic acids. In general, the nucleic acid is derived by the in-frame fusion of coding sequences from two or more proteins which have CDK-inhibitory motifs, such motifs being preserved in the resultant chimeric protein. Accordingly, such chimeric proteins can be derived to include, for example, CKI protein sequences, such as from INK4 or CIP proteins. For instance, as described in the appended examples, a coding sequence providing the CDK-binding motif of an INK4 protein can be fused in frame to a coding sequence providing a CDK-binding motif of a CIP protein.

Exemplary nucleic acid of the present invention encode fusion proteins which include at least a CDK-binding portion of an INK4 protein, such as p15, p16, p18 or p19. In preferred embodiments, the chimeric protein includes at least the two ankyrin-like sequence of the C-terminal portion of the INK4 protein, e.g. corresponding to the 3rd request (residues 69-101) and 4th repeat (residues 102-133) of $p16^{INK4A}$ (see Serrano et al. (1993) Nature 366:704-707).

Similarly, preferred chimeric proteins of the present invention include at least the p21/27-related inhibitory domain of a CIP protein, e.g. from p21, p27 or p57. For example, the chimeric protein can include the CDK-inhibitory motif corresponding to residues 28-79 of p27, residues 17-68 of p21, and/or residues 31-82 of p57, though larger fragments may be used such as described in the appended examples.

Moreover, CDK-binding motifs homologous to those occurring in either the INK4 or CIP protein families have been observed in other proteins. For example, the p21/p27-related inhibitory domain typical of the CIP protein family has been identified in such other proteins as the Rb-related protein p107 (Zhu et al.

(1995) Genes Dev 9:1740-1752). Likewise, ankyrin-like repeats homologous with the INK4 proteins have been identified in such other proteins as the Pho81p protein (Ogawa et al. (1995) Mol Cell Biol 15:997-1004). Consequently, it will be apparent to one of ordinary skill in the art, based on the disclosure herein, that functional equivalents of the INK4 and CIP proteins, e.g. which are capable of binding to a CDK and inhibiting kinase activation, exist and can be provided in the subject chimeric proteins.

Furthermore, it will be understood that the subject chimeric proteins can include CDK-binding motifs from proteins unrelated to either the INK4 family or CIP family. Moreover, such CDK-binding motifs, while inhibitory in and of themselves, can be derived from proteins which are otherwise activating in their full length form. To illustrate, the subject chimeric protein can be generated with a fragment of a cyclin which retains its CDK binding ability but not the CDK activating ability characteristic of the full length protein.

In some instances it may be necessary to introduce an unstructured polypeptide linker region between portions of the chimeric protein derived from different proteins. This linker can facilitate enhanced flexibility of the chimeric protein allowing the CDK-binding motifs from each portion to freely and (optionally) simultaneously interact with a CDK by reducing steric hindrance between the two fragments, as well as allowing appropriate folding of each portion to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence $(Gly_4Ser)_3$ can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent Nos. 5,091,513 and 5,258,498. Naturally occurring unstructured linkers of human origin are preferred as they reduce the risk of immunogenicity.

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to

complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a fusion gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992).

The term nucleic acid as used herein is intended to include nucleotide sequences encoding functionally equivalent chimeric proteins which, for example, retain the ability to bind to a cyclin-dependent kinase. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include sequences that differ from the nucleotide sequence of, for example, an INK4 or CIP gene known in the art due to the degeneracy of the genetic code. Equivalents will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to the nucleotide sequence encoding a naturally-occurring CDK-binding motif. Furthermore, equivalent nucleic acids will include those with nucleotide sequences which differ from the natural sequence which encodes a CDK-binding motif because of degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid can, accordingly, be used to replace codons in the naturally-occurring sequence.

This invention also provides expression vectors comprising a nucleotide sequence encoding a subject CDK inhibitor chimeric protein and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the fusion protein. Accordingly, the term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences-sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding the fusion proteins of this invention. Such useful expression control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes,

the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. Of course, the transcriptional regulatory sequences can include those sequences which naturally control expression of one of the genes used to derive the fusion protein, such as 5' flanking sequences of an INK4 or CIP gene.

It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

Expression vehicles for production of recombinant forms of the subject chimeric proteins include plasmids and other vectors. For instance, suitable vectors for expression of a fusion protein of the present invention include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as E. coli.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into S. cerevisiae (see, for example, Broach et al. (1983) in Experimental Manipulation of Gene Expression, ed. M. Inouye Academic Press, p. 83). These vectors can replicate in E. coli due the presence of the pBR322 ori, and in S. cerevisiae due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

The preferred mammalian expression vectors (other than for gene therapy) contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as

the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells.

In some instances, it may be desirable to express the subject fusion protein by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989) Chapters 16 and 17.

Another aspect of the present invention concerns preparations of the subject chimeric proteins. In particular, purified and semi-purified preparations of the CDK inhibitors can be formulated according to specifications attendant the desired use of the chimeric protein.

With respect to purifying the subject chimeric proteins, Applicant notes that it is widely appreciated that addition of certain heterologous sequences to a protein can facilitate the expression and purification of the proteins. For example, a fusion protein of the present invention can be generated to also include a glutathione-S-transferase (GST) polypeptide sequence. The GST portion of the recombinant proteins can enable easy purification of the protein, such as by the use of glutathione-derivativized matrices (see, for example, Current Protocols in Molecular Biology, eds. Ausabel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, the subject fusion protein can also include a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence located at the N-terminus of the subject fusion protein. Such sequences facilitates purification of the poly(His)-expressed fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) J. Chromatography 411:177; and Janknecht et al. PNAS 88:8972).

The present invention further pertains to methods of producing the subject chimeric proteins. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding one of the chimeric proteins of the present invention can be cultured under appropriate conditions to

allow expression of the polypeptide to occur. The peptide may be secreted and isolated from a mixture of host cells and medium by inclusion of a signal secretion sequence. Alternatively, the peptide may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant chimeric protein can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immuno-affinity purification with antibodies specific for portions of the chimeric protein.

This invention also pertains to a host cell transfected to recombinantly express one of the subject chimeric proteins. The host cell may be any prokaryotic or eukaryotic cell. Thus, a nucleic acid derived from the fusion of coding sequences for two or more CDK-binding motifs from different proteins can be used to produce a recombinant form of the chimeric protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g., p16, p21, p27, p57, p107, cyclins and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant chimeric proteins by microbial means or tissue-culture technology in accord with the subject invention.

The chimeric molecules of the present invention can also be generated using well-known cross-linking reagents and protocols. For example, there are a large number of chemical cross-linking agents that are known to those skilled in the art and useful for cross-linking two heterologous polypeptide chains. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link molecules in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N- hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-

succinimidyloxycarbonyl- a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage in vivo.

In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl suberate (DSS), bismaleimidohexane (BMH) and dimethylpimelimidate·2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[ß-(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl- amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this invention. For a recent review of protein coupling techniques, see Means et al. (1990) Bioconjugate Chemistry 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

Preparing protein-conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the protein chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl, usually from a cysteine residue. Free sulfhydryls can be generated by reduction of protein disulfides. Alternatively, a primary amine may be modified with Traut's

Reagent to add a sulfhydryl (Blattler et al. (1985) Biochem 24:1517, incorporated by reference herein). Again, Ellman's Reagent can be used to calculate the number of sulfhydryls available in protein.

In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing protein under the appropriate buffer conditions. The protein-protein conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

For certain of the therapeutic uses of the subject chimeric proteins, particularly cutaneous uses such as for the control of keratinocyte proliferation, direct administration of the protein will be appropriate (rather than use of a gene therapy construct). Accordingly, the subject chimeric protein, or a pharmaceutically acceptable salt thereof, may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. In preferred embodiments, the chimeric protein is dispersed in lipid formulations, such as miscelles, which closely resemble the lipid composition of natural cell membranes to which the chimeric protein is to be delivered.

The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the chimeric protein, its use in the pharmaceutical preparation of the invention is contemplated. Suitable v hicles and their formulation inclusive of other proteins are described. for

example, in the book Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985).

In an exemplary embodiment, the chimeric protein is provided for transmucosal or transdermal delivery. For such administration, penetrants appropriate to the barrier to be permeated are used in the formulation with the polypeptide. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the proteins of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

Yet another aspect of the invention pertains to methods of treating proliferative and/or differentiative disorders which arise from cells which, despite aberrant growth control, still require one or more CDKs (e.g., CDK4 or CDK6) for cell growth. There are a wide variety of pathological cell proliferative conditions for which the fusion gene constructs of the present invention can provide therapeutic benefits, with the general strategy being the inhibition of an anomalous cell proliferation. For instance, the gene constructs of the present invention can be used as a part of a gene therapy protocol in a cell in which a cell-cycle regulatory protein (such as an INK4 or CIP protein) is misexpressed or in which signal transduction pathways upstream of the protein are dysfunctional. To illustrate, cell types which exhibit pathological or abnormal growth presumably dependent at least in part on a function of a, INK4 or CIP protein include various cancers and leukemias, psoriasis, bone diseases, fibroproliferative disorders such as involving connective tissues, atherosclerosis and other smooth muscle proliferative disorders, as well as chronic inflammation. In addition to proliferative disorders, the treatment of differentiative disorders which result from, for example, de-differentiation of tissue which may (optionally) be accompanied by abortive reeentry into mitosis. Such degenerative disorders include chronic neurodegenerative diseases of the nervous system, including Alzheimer's disease, Parkinson's disease, Huntington's chorea, amylotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations. Other differentiative disorders include, for example, disorders associated with connective tissue, such as may occur due to de-differentiation of chondrocytes or osteocytes, as well as vascular disorders which involve de-differentiation of endothelial tissue and smooth muscle cells, gastric ulcers characterized by degenerative changes in glandular cells, and renal conditions

marked by failure to differentiate, e.g. Wilm's tumors. It will also be apparent that, by transient use of gene therapy constructs of the subject fusion proteins, in vivo reformation of tissue can be accomplished, e.g. in the development and maintenance of organs. By controlling the proliferative and differentiative potential for different cells, the subject gene constructs can be used to reform injured tissue, or to improve grafting and morphology of transplanted tissue. For example, the subject CDK inhibitors can be employed therapeutically as part of a regimen to regulate organs after physical, chemical or pathological insult.

Furthermore, as described in the art, transformation of a cell can be due in part to a loss-of-function mutation to a particular INK4 gene, e.g., ranging from a point mutation to gross deletion of the gene. Additionally, other data suggests that certain disorders may arise because cells have lost the ability to induce expression of an INK4 gene. Normal cell proliferation, for instance, is generally marked by responsiveness to negative autocrine or paracrine growth regulators, such as members of the TGF-β family, e.g. TGF-β1, TGF-β2 or TGF-β3, and related polypeptide growth inhibitors. Ordinarily, control of cellular proliferation by such growth regulators, particularly in epithelial and hemopoietic cells, is in the form of growth inhibition. Moreover, as described in Hannon and Beach (1995) Nature 371:257-261, TGF-β inhibits cell proliferation by inducing expressions of p15, which in turn inhibits activation of CDK4 or CDK6 complexes.

It has been observed that a significant percentage of human cancers derived from cells types ordinarily inhibited by TGF-β display a reduced responsiveness to this growth regulator. For instance, some tumors of colorectal, liver epithelial, and epidermal origin show reduced sensitivity and resistance to the growth-inhibitory effects of TGF-β as compared to their normal counterparts. In this context, a noteworthy characteristic of several retinoblastoma cell lines is the absence of detectable TGF-β receptors. Treatment of such tumors with the subject fusion proteins provides an opportunity to mimic the TGF-β inhibitory signal. Moreover, it will be appreciated that the subject method can be used generally to inhibit proliferation of cells which, in general, are still reliant on cyclin dependent kinases.

In accordance with the subject method, expression constructs of the subject fusion proteins may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells in vivo with a recombinant fusion gene. Approaches include insertion of the subject fusion gene in viral vectors including recombinant retroviruses, adenovirus, adeno-

associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out in vivo. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for in vivo introduction of nucleic acid encoding one of the subject fusion proteins into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding one of the subject CCR-proteins, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-

9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

In choosing retroviral vectors as a gene delivery system for the subject fusion proteins, it is important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the recombinant gene, is that the target cells must be dividing. In general, this requirement will not be a hindrance to use of retroviral vectors to deliver the subject fusion gene constructs. In fact, such limitation on infection can be beneficial in circumstances where the tissue (e.g. nontransformed cells) surrounding the target cells does not undergo extensive cell division and is therefore refractory to infection with retroviral vectors.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al. (1989) PNAS 86:9079-9083; Julan et al. (1992) J. Gen Virol 73:3251-3255; and Goud et al. (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al. (1991) J Biol Chem 266:14143-

14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the fusion gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilitizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) Cell 16:683; Berkner et al., supra; and Graham et al. in

Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted fusion gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject fusion gene is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistent expression of the subject fusion proteins in cells of the central nervous system and ocular tissue (Pepose et al. (1994) Invest Ophthalmol Vis Sci 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a the subject fusion proteins in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding one of the subject fusion proteins can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) No Shinkei Geka 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal antibodies against glioma-associated antigen (Mizuno et al. (1992) Neurol. Med. Chir. 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject gene construct can be used to transfect hepatocytic cells in vivo using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al. (1993) Science 260-926; Wagner et al. (1992) PNAS 89:7934; and Christiano et al. (1993) PNAS 90:2122).

In clinical settings, the gene delivery systems can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057).

Moreover, the pharmaceutical preparation can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced in tact from recombinant cells, e.g. retroviral packages, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system. In the case of the latter, methods of introducing the viral packaging cells may be provided by, for example, rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested in vivo in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals, and can be adapted for release of viral particles through the manipulation of the polymer composition and form. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of an the viral particles by cells implanted at a particular target site. Such embodiments of the present invention can be used for the delivery of an exogenously purified virus, which has been incorporated in the polymeric device, or for the delivery of viral particles produced by a cell encapsulated in the polymeric device.

By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, Concise Encyclopedia of Medical & Dental Materials, ed. by David Williams (MIT Press: Cambridge, MA, 1990); and the Sabel et al. U.S. Patent No. 4,883,666. In another embodiment of an implant, a source of cells producing the recombinant virus is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the viral source (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the viral packaging cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotechnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials

12:50-55). Again, manipulation of the polymer can be carried out to provide for optimal release of viral particles.

To further illustrate the use of the subject method, the therapeutic application of a CDK inhibitor fusion protein, e.g., by gene therapy, can be used in the treatment of a neuroglioma. Gliomas account for 40-50% of intracranial tumors at all ages of life. Despite the increasing use of radiotherapy, chemotherapy, and sometimes immunotherapy after surgery for malignant glioma, the mortality and morbidity rates have not substantially improved. However, there is increasing experimental and clinical evidence that for a significant number of gliomas, loss of TGF-β responsiveness is an important event in the loss of growth control. Irrespective of the cause of decreased responsiveness, e.g. the loss of function of p15 or the loss of other TGF-β signal transduction proteins, exogenous expression of, for example, an INK4 fusion protein such as p15/p27 fusion protein in the cell can used effectively to inhibit cell proliferation.

It has been demonstrated that gene therapy can be used to target glioma cells for expression of recombinant proteins (Miyao et al. (1993) J. Neurosci. Res. 36:472-479; Chen et al. (1994) PNAS 91:3054-3057; and Takamiya et al. (1993) J. Neurosurg. 79:104-110). Thus, a gene construct for expressing the subject fusion protein can be delivered to the tumor, preferably by sterotactic-dependent means. In preferred embodiments, the gene delivery system is a retroviral vector. Since rapidly growing normal cells are rare in the adult CNS, glioma cells can be specifically transduced with a recombinant retrovirus. For example, the retroviral particle can be delivered into the tumor cavity through an Ommaya tube after surgery, or alternatively, packaging fibroblasts encapsulated in retrievable immunoisolatory vehicles can be introduced into the tumor cavity. In order to increase the effectiveness and decrease the side effects of the retrovirus-mediated gene therapy, glioma-specific promoters can be used to regulate expression of the therapeutic gene. For example, the promoter regions of glial fibrillary acidic protein (GFAP) and myelin basis protein (MBP) can operably linked to the fusion gene in order to direct glial cell-specific expression of the fusion protein.

In another embodiment, gene therapy can be used in conjunction with the subject fusion proteins in the treatment of various carcinomas. In a representative embodiment, a gene therapy system comprising the subject fusion gene is used to treat certain breast cancers. In preferred embodiments, expression of the subject fusion protein is controlled at least in part by a mammary-specific

promoter, a number of which are available (for review, see Hennighausen (1990) Protein Expression and Purification 1:3-8; and Günzberg et al. (1992) Biochem J 283:625-632).

In similar fashion, gene therapy protocols involving delivery of the subject fusion protein can be used in the treatment of malignant melanoma, which also serves as a model for progressive TGF-β resistance in transformation. In preferred embodiments, gene therapy protocols for treatment of melanomas include, in addition to the delivery of the fusion gene construct, the delivery of a pharmaceutical preparation of the gene by direct injection. For instance, U.S. patent no. 5,318,514 describes an applicator for the electroporation of genes into epidermal cells and can be used in accordance with the present invention.

The subject fusion proteins can be used in the treatment of hyperproliferative vascular disorders, e.g. smooth muscle hyperplasia (such as atherosclerosis) or restinosis, as well as other disorders characterized by fibrosis, e.g. rheumatoid arthritis, insulin dependent diabetes mellitus, glomerulonephritis, cirrhosis, and scleroderma, particularly proliferative disorders in which loss of TGF-β autocrine or paracrine signaling, and accordingly loss of p15 function, is implicated.

For example, restinosis continues to limit the efficacy of coronary angioplasty despite various mechanical and pharmaceutical interventions that have been employed. An important mechanism involved in normal control of intimal proliferation of smooth muscle cells appears to be the induction of autocrine and paracrine TGF-β inhibitory loops in the smooth muscle cells (Scott-Burden et al. (1994) Tex Heart Inst J 21:91-97; Graiger et al. (1993) Cardiovasc Res 27:2238-2247; and Grainger et al. (1993) Biochem J 294:109-112). Loss of sensitivity to TGF-β, or alternatively, the overriding of this inhibitory stimulus such as by PDGF autostimulation, can be a contributory factor to abnormal smooth muscle proliferation in restinosis. It may therefore be possible to treat or prevent restinosis by the use of gene therapy with CDK inhibitor fusion protein of the present invention. The fusion gene construct can be delivered, for example, by percutaneous transluminal gene transfer (Mazur et al. (1994) Tex Heart Inst J 21:104-111) using viral or liposomal delivery compositions. An exemplary adenovirus-mediated gene transfer technique and compositions for treatment of cardiac or vascular smooth muscle is provided in PCT publication WO 94/11506.

Transforming growth factor-β is also understood to play a significant role in local glomerular and interstitial sites in human kidney development and

disease. Consequently, the subject method provides a method of treating or inhibiting glomerulopathies and other renal proliferative disorders comprising the in vivo delivery and recombinant expression of the subject fusion proteins in kidney tissue.

The subject method can also be used to treat retinoblastomas in which the retinoblastoma gene (RB) is not itself impaired, e.g. the effective impairment of the RB checkpoint is the result of a failure to control CDK4 phosphorylation of RB. Thus, one of the subject fusion proteins can be expressed in a retinoblastoma cell, thereby causing inhibition of CDK4 activation and down-regulating RB phosphorylation. To illustrate, a recombinant retrovirus can be constructed to facilitate expression of a fusion protein including an INK4 protein, e.g., derived from p16 or p15, and a CIP protein, e.g., derived from p21, p27 or p57. Infectivity of retinoblastoma cells can be enhanced by derivatizing the env protein with antibodies specific for retinoblastoma cells, e.g. antibodies to retinal S-antigen (Doroso et al. (1985) Invest Opthalmol Vis Sci 26:560-572; see also Liao et al. (1981) Eur J Immunol 11:450-454; and U.S. Patent No. 4,444,744).

In yet another embodiment, the subject gene is delivered to a sarcoma, e.g. an osteosarcoma or Kaposi's sarcoma. In a representative embodiment, the gene is provided in a viral vector and delivered by way of a viral particle which has been derivatized with antibodies immunoselective for an osteosarcoma cell (see, for example, U.S. Patents 4,564,517 and 4,444,744; and Singh et al. (1976) Cancer Res 36:4130-4136).

Given the role of CDK activation in various epithelial cell proliferative disorders, it will be evident that the subject fusion proteins will find ready application for the treatment or prophylaxis of, for example, psoriasis; keratosis; acne; comedogenic lesions; verrucous lesions such as verruca plana, plantar warts, verruca acuminata, and other verruciform lesions marked by proliferation of epithelial cells; folliculitis and pseudofolliculitis; keratoacanthoma; callosities; Darier's disease; ichthyosis; lichen planus; molluscous contagiosum; melasma; Fordyce disease; and keloids or hypertrophic scars.

Yet another aspect of the present invention relates to the use of the subject fusion proteins to control hair growth. The growth of hard keratin fibers such as wool and hair is dependent on the proliferation of dermal sheath cells. Hair follicle stem cells of the sheath are highly active, and give rise to hair fibers through rapid proliferation and complex differentiation. The hair cycle involves three distinct phases: anagen (growing), catagen (regressing), and telogen (resting). The

epidermal stem cells of the hair follicle are activated by dermal papilla during late telogen. This is termed "bulge activation". Moreover, such stem cells are thought to be pluripotent stem cells, giving rise not only to hair and hair follicle structures, but also the sebaceous gland and epidermis. The subject method provides a means for altering the dynamics of the hair growth cycle to induce quiescence of proliferation of hair follicle cells, particularly stem cells of the hair follicle, inhibiting CDK activation.

For instance, gene therapy treatments or, alternatively, topical administration of a fusion protein preparation, can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g. hypertrichosis. In an exemplary embodiment, the subject fusion proteins can be used to manage hirsutism, a disorder marked by abnormal hairiness. Application of the CDK inhibitors of the present invention can also provide a process for extending the duration of depilation.

Moreover, because the CDK inhibitor fusion proteins are likely to be cytostatic to epithelial cells, rather than cytotoxic, these proteins can be used to protect hair follicle cells from cytotoxic agents which require progression into S-phase of the cell-cycle for efficacy, e.g. radiation-induced death. Treatment with a CDK inhibitor of the present invention provides protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, such treatments can be used for patients undergoing chemo- or radiation-therapies which ordinarily result in hair loss.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematosa reticulata or keloid folliculitis. For example, a cosmetic prepration of an CDK inhibitory fusion protein can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In similar fashion, such preparations can be used in the treatment of granulomas, e.g. tumor-like mass or nodule of granulation tissue, which may include epithelial tissue derived from cutaneous or mucosal sources.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which inhibition of epithelial cell proliferation in and around periodontal tissue is desired. For example, preparations of the present invention can find application in the treatment of peridontal disease. It is estimated that in the United States alone, there are in excess of 125 million adults with periodontal disease in varying forms. Periodontal disease starts as inflammatory lesions because of specific bacteria localizing in the area where the gingiva attaches to the tooth. Usually first to occur is a vascular change in the underlying connective tissue. Inflammation in the connective tissue stimulates the following changes in the epithelial lining of the sulcus and in the epithelial attachment: increased mitotic activity in the basal epithelial layer; increased producing of keratin with desquamation; cellular desquamation adjacent to the tooth surface tends to deepen the pocket; epithelial cells of the basal layer at the bottom of the sulcus and in the area of attachment proliferate into the connective tissue and break up of the gingival fibers begins to occur, wherein dissolution of the connective tissue results in the formation of an open lesion. The application of CDK inhibitor preparations to the periodontium can be used to inhibit proliferation of epithelial tissue and thus prevent further periodontoclastic development.

In yet another embodiment of the present invention, the subject CDK inhibitors can be used to inhibit spermatogenesis or oogenesis by inhibiting progression through mitotic or meiotic cell-cycle stages. The anti-mitotic and/or anti-meiotic activity of the fusion proteins identified in the present invention may accordingly be used, for example, in birth control methods by disrupting oogenic pathways in order to prevent the development of either the egg or sperm, or by preventing mitotic progression of a fertilized egg.

In a still further embodiment, the subject fusion protein is recombinantly expressed in tissue which is characterized by unwanted de-differentiation and which may also be undergoing unwanted apoptosis. For instance, many neurological disorders are associated with degeneration of discrete populations of neuronal elements. For example, Alzheimer's disease is associated with deficits in several neurotransmitter systems, both those that project to the neocortex and those that reside with the cortex. For instance, the nucleus basalis in patients with Alzheimer's disease were observed to have a profound (75%) loss of neurons compared to age-matched controls. Although Alzheimer's disease is by far the most common form of dementia, several other disorders can produce dementia. Many are age-related, occurring in far greater incidence in older people than in

younger. Several of these are degenerative diseases characterized by the death of neurons in various parts of the central nervous system, especially the cerebral cortex. However, some forms of dementia are associated with degeneration of the thalamus or the white matter underlying the cerebral cortex. Here, the cognitive dysfunction results from the isolation of cortical areas by the degeneration of efferents and afferents. Huntington's disease involves the degeneration of intrastriatal and cortical cholinergic neurons and GABAergic neurons. Pick's disease is a severe neuronal degeneration in the neocortex of the frontal and anterior temporal lobes, sometimes accompanied by death of neurons in the striatum. Accordingly, the subject fusion proteins can be delivered to the effected tissue by gene therapy techniques. It is noted that numerous advances have been made in the construction of expression vectors, cellular and viral transgene carriers, and the characterization of target cells for neuronal gene therapy, and can be readily adapted for delivery of the subject genes (see, for example, Suhr et al. (1993) Arch Neurol 50:1252-1268; Jiao et al. (1993) Nature 362:450-453; Friedmann (1992) Ann Med 24:411-417; and Freese et al. (1991) Nuc Acid Res 19:7219-7223)

In addition to degenerative-induced dementias, the subject gene therapy systems can be applied opportunely in the treatment of neurodegenerative disorders which have manifestations of tremors and involuntary movements. Parkinson's disease, for example, primarily affects subcortical structures and is characterized by degeneration of the nigrostriatal pathway, raphe nuclei, locus cereleus, and the motor nucleus of vagus. Ballism is typically associated with damage to the subthalmic nucleus, often due to acute vascular accident. Also included are neurogenic and myopathic diseases which ultimately affect the somatic division of the peripheral nervous system and are manifest as neuromuscular disorders. Examples include chronic atrophies such as amyotrophic lateral sclerosis, Guillain-Barre syndrome and chronic peripheral neuropathy, as well as other diseases which can be manifest as progressive bulbar palsies or spinal muscular atrophies. Moreover, the use of the subject fusion gene therapy constructs is amenable to the treatment of disorders of the cerebellum which result in hypotonia or ataxia, such as those lesions in the cerebellum which produce disorders in the limbs ipsilateral to the lesion. For instance, p16/p27 fusion gene constructs can used to treat a restricted form of cerebellar cortical degeneration involving the anterior lobes (vermis and leg areas) such as is common in alcoholic patients.

Furthermore, the subject fusion proteins can also be used in the treatment of autonomic disorders of the peripheral nervous system, which include

disorders affecting the innervation of smooth muscle and endocrine tissue (such as glandular tissue). For instance, recombinant fusion protein of the present invention can be expressed by gene therapy and used to treat tachycardia or atrial cardiac arrythmias which may arise from a degenerative condition of the nerves innervating the striated muscle of the heart.

As will be apparent, the subject gene constructs can be used to cause expression of the fusion polypeptides in cells propagated in culture, e.g. to produce proteins or polypeptides, including fusion proteins or polypeptides, for purification. In addition, recombinant expression of the subject fusion polypeptides in cultured cells can be useful for controlling differentiation states of cells in vitro, for instance, by controlling the level of activation of a CDK. To illustrate, in vitro neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors. Once a neuronal cell has become terminally-differentiated, it typically will not change to another terminally differentiated cell-type. However, neuronal cells can nevertheless readily lose their differentiated state. This is commonly observed when they are grown in culture from adult tissue, and when they form a blastema during regeneration. By preventing the activation of one or more CDKs, particularly in G_0 or G_1, certain of the subject fusion proteins can prevent mitotic progression and hence provide a means for ensuring an adequately restrictive environment in order to maintain neuronal cells at various stages of differentiation, and can be employed, for instance, in cell cultures designed to test the specific activities of trophic factors. Other tissue culture systems which require maintenance of differentiation will be readily apparent to those skilled in the art. In this respect, each of the subject antagonist of CDK4 activation can be used for ex vivo tissue generation, as for example, to enhance the generation of prosthetic tissue devices for implantation. That is, by inhibiting the activation of a CDK with one of the subject fusion proteins, cultured cells can be guided along certain differentiative pathways.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Prototype embodiments of the CDK inhibitory fusion protein described above were derived from the fusion of the coding sequences from the human p27 and p16 cDNAs. The nucleotide sequence for the fusion gene encoding the p27-p16 protein is provided in SEQ ID No. 1, with the corresponding amino acid sequence being designated by SEQ ID No. 2. The construct includes a poly(His) leader for purification, along with a hinge region including a $(Gly_4Ser)_3$ linker to permit proper folding and breathing of each of the p27 and p16 portions of the resulting protein. The sequences for both human p27 and human p16 have been described in the art. Briefly, the p27-p16 fusion protein was constructed as follow.

The expression vector is pT7-7 from US Biocehmical. To construct the p27-p16 fusion, first we PCR amplified the p27 coding sequence using the following primers:

N-terminal primer: (SEQ ID No. 3)
5'-GCGGCCGGTCATATGCACCACCATCACCATCACTCAAACG-TGCGAGTGTCT-3'
This primer carries an Ndel site and 6 histidine codons that are inserted between the ATG and the second amino acid of p27.

C-terminal primer: (SEQ ID No. 4)
5'-
GCCGCCGGCGTCGACTCGGCCGAATTCGGATCCACCCCCGCCGGAACC-GCCACCCCCGCTGCCCCCGCCACCCGTTTGACGTCTTCTGAGGCCAGG-3'
This primer carries the $(Gly_4Ser)_3$ repeat and EcoR1, Sal1 and Hind3 restriction sites and eliminates the stop codon of p27.

The p27 PCR product was cut with Ndel and Hind3 and inserted into pT7-7 cut with Ndel and Hind3. The resulted construct was cut with EcoR1 and Sal1 and a full length p16 PCR product was inserted as an EcoR1-Xho1 fragment. The position of the EcoR1 site allows the in-frame insertion of p16. The rest of the hinge region between the p27 and p16 coding sequences derives from the 5' end of the p16 cDNA.

The pT7p27-p16 expression plasmid was transformed into BL21 cells. For fusion protein expression, cells were grown in LB + 50μg/ml ampicillin at 37C to OD_{600}=0.8 and protein expression was induced by IPTG (final; conc.: 20mM) for 4 hours as 37C. Cells were collected and the pellet was frozen at -80C. The preparation of the cell lysate and binding to a Ni^{2+} charged sepharose resin

(Invitrogen catalog no. R801) was done according to the manufacturer's instruction (Invitrogen; see also Hochuli et al. (1987) J. Chromatography 411:177-184; and Janknecht et al. (1991) PNAS 88:8972-8976). The bound proteins were eluted with 50mM, 200mM, 350mM, and 500mM imidazol and the fractions were analyzed on SDS/PAGE. The 200mM, 350mM, and 500mM imidazol fractions were collected, dialised against 1xPBS(1mM KH_2PO_4, 10mM Na_2HPO_4, 137mM NaCl, 2.7mM KCl, pH=7.4) + 10% glycerol and stored at -80C in aliquots. ~25% of the prep was the fusion protein.

The purity of the p27-p16, p27, and p16 preperationss were normalized using p16 and p27 specific antibodies.

The kinase inhibitory activity of the p27-p16 fusion protein was determined using an in vitro kinase assay in which the kinase activity of a particular cyclin/CDK complex was measured for varying concentrations of fusion protein. Briefly, the assay employs Sf9 cell extracts that were made from cells that were coinfected with the proper CDK and cyclin expression constructs. Typically, 44µg of Sf9 extract in 50µl of 50mM Tris/Cl pH=7.6, 10mM $MgCl_2$, 1mM DTT, 25µM ATP, 10µCi ^{32}P-γ-ATP was used in the absence of the presnce of the particular inhibitor (inhibitor concentration was between 25nM to 1µM). The reaction was carried out at 30°C for 30 minutes using 2µg of Gst-Rb as a substrate. Gst-Rb was recaptured using GSH-agaraose, separated on 10% SDS/PAGE and stained with Comassie blue. After autoradiography the GST-Rb bands were cut out and ^{32}P incorporation was measured.

The concentration of p27-p16 fusion protein at which 50% of the kinase activity was blocked (IC_{50}) was calculated for various cyclin/CDK pairs. The results are indicated in Table I.

Table I

Inhibition of cyclin dependent kinase complexes by p27-p16 fusion protein

inhibitor	CDK4/cycl in D1	CDK2/cycl in E	CDK2/cycl in A	cdc2/cyclin B
p27-p16	25 nm	30 nm	25 nm	15 nm
p27	63 nm	52 nm	65 nm	20 nm
p16	250 nm	>500 nm	>500 nm	>500 nm

nm=nanomolar

Moreover, the inhibition constant, K_i for the inhibition of CDK4/cyclin D1 by p27-p16 fusion protein was determined to be 23 nm, compared to a K_i of 75 nm for p16 inhibition of the same CDK4 complex.

Other exemplary fusion proteins were derived as follows:
(i) a "p16(GS)p27" fusion protein was generated to include, N to C terminal, the entire coding sequence of p16, fused in frame with a $(Gly_4Ser)_3$ linker and then the full coding sequence of p27. The nucleotide sequence for the fusion gene encoding the p16(GS)p27 protein is provided in SEQ ID No. 4, with the corresponding amino acid sequence being designated by SEQ ID No. 5; and
(ii) a "p16p27" fusion protein was generated to include, N to C terminal, the entire coding sequence of p16, fused in frame the full coding sequence of p27 (no $(Gly_4Ser)_3$ linker). The nucleotide sequence for the fusion gene encoding the p16p27 protein is provided in SEQ ID No. 6, with the corresponding amino acid sequence being designated by SEQ ID No. 7.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: INHIBITORS OF CELL-CYCLE PROGRESSION, AND USES RELATED THERETO

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US97/
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: USSN 08/589,981
(B) FILING DATE: 23 JANUARY 1996

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1420 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 4..1176

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 4..24
(D) OTHER INFORMATION: /label= POLY-HIS_TAG

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAT ATG CAC CAC CAT CAC CAT CAC TCA AAC GTG CGA GTG TCT AAC GGG      48
    Met His His His His His His Ser Asn Val Arg Val Ser Asn Gly
      1               5                  10                  15

AGC CCT AGC CTG GAG CGG ATG GAC GCC AGG CAG GCG GAG CAC CCC AAG      96
Ser Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys
                  20                  25                  30

CCC TCG GCC TGC AGG AAC CTC TTC GGC CCG GTG GAC CAC GAA GAG TTA     144
Pro Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu
              35                  40                  45

ACC CGG GAC TTG GAG AAG CAC TGC AGA GAC ATG GAA GAG GCG AGC CAG     192
Thr Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln
          50                  55                  60

CGC AAG TGG AAT TTC GAT TTT CAG AAT CAC AAA CCC CTA GAG GGC AAG     240
Arg Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys
      65                  70                  75

TAC GAG TGG CAA GAG GTG GAG AAG GGC AGC TTG CCC GAG TTC TAC TAC     288
Tyr Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr
  80                  85                  90                  95

AGA CCC CCG CGG CCC CCC AAA GGT GCC TGC AAG GTG CCG GCG CAG GAG     336
Arg Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu
                 100                 105                 110

AGC CAG GAT GTC AGC GGG AGC CGC CCG GCG GCG CCT TTA ATT GGG GCT     384
Ser Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala
             115                 120                 125

CCG GCT AAC TCT GAG GAC ACG CAT TTG GTG GAC CCA AAG ACT GAT CCG     432
Pro Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro
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          130                 135                 140

TCG GAC AGC CAG ACG GGG TTA GCG GAG CAA TGC GCA GGA ATA AGG AAG      480
Ser Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys
    145                 150                 155

CGA CCT GCA ACC GAC GAT TCT TCT ACT CAA AAC AAA AGA GCC AAC AGA      528
Arg Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg
160                 165                 170                 175

ACA GAA GAA AAT GTT TCA GAC GGT TCC CCA AAT GCC GGT TCT GTG GAG      576
Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu
                180                 185                 190

CAG ACG CCC AAG AAG CCT GGC CTC AGA AGA CGT CAA ACG GGT GGC GGG      624
Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr Gly Gly Gly
            195                 200                 205

GGC AGC GGG GGT GGC GGT TCC GGC GGG GGT GGA TCC GAA TTC TGC GGC      672
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Phe Cys Gly
        210                 215                 220

CGC GCG TGC GCT CGG CGG CTG CGG AGA GGG GAG AGC ATG CAG CGG GCG      720
Arg Ala Cys Ala Arg Arg Leu Arg Arg Gly Glu Ser Met Gln Arg Ala
    225                 230                 235

GCG GGG AGC AGC ATG GAG CCT TCG GCT GAC TGG CTG GCC ACG GCC GCG      768
Ala Gly Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala
240                 245                 250                 255

GCC CGG GGT CGG GTA GAG GAG GTG CGG GCG CTG CTG GAG GCG GTG GCG      816
Ala Arg Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Val Ala
                260                 265                 270

CTG CCC AAC GCA CCG AAT AGT TAC GGT CGG AGG CCG ATC CAG GTC ATG      864
Leu Pro Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met
            275                 280                 285

ATG ATG GGC AGC GCC CGA GTG GCG GAG CTG CTG CTG CTC CAC GGC GCG      912
Met Met Gly Ser Ala Arg Val Ala Glu Leu Leu Leu His Gly Ala
        290                 295                 300

GAG CCC AAC TGC GCC GAC CCC GCC ACT CTC ACC CGA CCC GTG CAC GAC      960
Glu Pro Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp
    305                 310                 315

GCT GCC CGG GAG GGC TTC CTG GAC ACG CTG GTG GTG CTG CAC CGG GCC     1008
Ala Ala Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala
320                 325                 330                 335

GGG GCG CGG CTG GAC GTG CGC GAT GCC TGG GGC CGT CTG CCC GTG GAC     1056
Gly Ala Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp
                340                 345                 350

CTG GCT GAG GAG CTG GGC CAT CGC GAT GTC GCA CGG TAC CTG CGC GCG     1104
```

```
Leu Ala Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala
           355                 360                 365

GCT GCG GGG GGC ACC AGA GGC AGT AAC CAT GCC CGC ATA GAT GCC GCG    1152
Ala Ala Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala
        370                 375                 380

GAA GGT CCC TCA GAC ATC CCC GAT TGAAAGAACC AGAGAGGCTC TGAGAAACCT   1206
Glu Gly Pro Ser Asp Ile Pro Asp
    385                 390

CGGGAAACTT AGATCATCAG TCACCGAAGG TCCTACAGGG CCACAACTGC CCCCGCCACA  1266

ACCCACCCCG CTTTCGTAGT TTTCATTTAG AAAATAGAGC TTTTAAAAAT GTCCTGCCTT  1326

TTAACGTAGA TATAAGCCTT CCCCCACTAC CGTAAATGTC CATTTATATC ATTTTTTATA  1386

TATTCTTATA AAAATGTAAA AAAGAAAACT CGAG                              1420
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 391 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met His His His His His His Ser Asn Val Arg Val Ser Asn Gly Ser
  1               5                  10                  15

Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys Pro
             20                  25                  30

Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu Thr
         35                  40                  45

Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln Arg
     50                  55                  60

Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys Tyr
 65                  70                  75                  80

Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg
                 85                  90                  95

Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu Ser
            100                 105                 110

Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro
        115                 120                 125
```

```
Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro Ser
    130                 135                 140

Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg
145                 150                 155                 160

Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr
                165                 170                 175

Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu Gln
            180                 185                 190

Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr Gly Gly Gly Gly
        195                 200                 205

Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Phe Cys Gly Arg
    210                 215                 220

Ala Cys Ala Arg Arg Leu Arg Arg Gly Glu Ser Met Gln Arg Ala Ala
225                 230                 235                 240

Gly Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala
                245                 250                 255

Arg Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Val Ala Leu
            260                 265                 270

Pro Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met
        275                 280                 285

Met Gly Ser Ala Arg Val Ala Glu Leu Leu Leu Leu His Gly Ala Glu
    290                 295                 300

Pro Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala
305                 310                 315                 320

Ala Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly
                325                 330                 335

Ala Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu
            340                 345                 350

Ala Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala
        355                 360                 365

Ala Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu
    370                 375                 380

Gly Pro Ser Asp Ile Pro Asp
385                 390
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGGCCGGTC ATATGCACCA CCATCACCAT CACTCAAACG TGCGAGTGTC T 51

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCGCCGGCG TCGACTCGGC CGAATTCGGA TCCACCCCCG CCGGAACCGC CACCCCCGCT 60

GCCCCCGCCA CCCGTTTGAC GTCTTCTGAG GCCAGG 96

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1143 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1140

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG GGA TAC CCT TAT GAT GTG CCA GAT TAT GCC GAT CCG GCG GCG GGG 48
Met Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Asp Pro Ala Ala Gly
1 5 10 15

```
AGC AGC ATG GAG CCT TCG GCT GAC TGG CTG GCC ACG GCC GCG GCC CGG      96
Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala Arg
             20                  25                  30

GGT CGG GTA GAG GAG GTG CGG GCG CTG CTG GAG GCG GGG GCG CTG CCC     144
Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Gly Ala Leu Pro
         35                  40                  45

AAC GCA CCG AAT AGT TAC GGT CGG AGG CCG ATC CAG GTC ATG ATG ATG     192
Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met Met
     50                  55                  60

GGC AGC GCC CGA GTG GCG GAG CTG CTG CTG CTC CAC GGC GCG GAG CCC     240
Gly Ser Ala Arg Val Ala Glu Leu Leu Leu Leu His Gly Ala Glu Pro
 65                  70                  75                  80

AAC TGC GCC GAC CCC GCC ACT CTC ACC CGA CCC GTG CAC GAC GCT GCC     288
Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala Ala
                 85                  90                  95

CGG GAG GGC TTC CTG GAC ACG CTG GTG GTG CTG CAC CGG GCC GGG GCG     336
Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly Ala
            100                 105                 110

CGG CTG GAC GTG CGC GAT GCC TGG GGC CGT CTG CCC GTG GAC CTG GCT     384
Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala
        115                 120                 125

GAG GAG CTG GGC CAT CGC GAT GTC GCA CGG TAC CTG CGC GCG GCT GCG     432
Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Ala
    130                 135                 140

GGG GGC ACC AGA GGC AGT AAC CAT GCC CGC ATA GAT GCC GCG GAA GGT     480
Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly
145                 150                 155                 160

CCC TCA GAC ATC CCC GAT GGT GGC GGG GGC AGC GGG GGT GGC GGT TCC     528
Pro Ser Asp Ile Pro Asp Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
                165                 170                 175

GGC GGG GGT GGA TCC GTC GAG TCA AAC GTG CGA GTG TCT AAC GGG CGC     576
Gly Gly Gly Gly Ser Val Glu Ser Asn Val Arg Val Ser Asn Gly Arg
            180                 185                 190

CCT AGC CTG GAG CGG ATG GAC GCC AGG CAG GCG GAG CAC CCC AAG CCC     624
Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys Pro
        195                 200                 205

TCG GCC TGC AGG AAC CTC TTC GGC CCG GTG GAC CAC GAA GAG TTA ACC     672
Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu Thr
    210                 215                 220

CGG GAC TTG GAG AAG CAC TGC AGA GAC ATG GAA GAG GCG AGC CAG CGC     720
Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln Arg
```

```
225                 230                 235                 240

AAG TGG AAT TTC GAT TTT CAG AAT CAC AAA CCC CTA GAG GGC AAG TAC    768
Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys Tyr
                245                 250                 255

GAG TGG CAA GAG GTG GAG AAG GGC AGC TTG CCC GAG TTC TAC TAC AGA    816
Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg
            260                 265                 270

CCC CCG CGG CCC CCC AAA GGT GCC TGC AAG GTG CCG GCG CAG GAG AGC    864
Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu Ser
        275                 280                 285

CAG GAT GTC AGC GGG AGC CGC CCG GCG GCG CCT TTA ATT GGG GCT CCG    912
Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro
    290                 295                 300

GCT AAC TCT GAG GAC ACG CAT TTG GTG GAC CCA AAG ACT GAT CCG TCG    960
Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro Ser
305                 310                 315                 320

GAC AGC CAG ACG GGG TTA GCG GAG CAA TGC GCA GGA ATA AGG AAG CGA   1008
Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg
                325                 330                 335

CCT GCA ACC GAC GAT TCT TCT ACT CAA AAC AAA AGA GCC AAC AGA ACA   1056
Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr
            340                 345                 350

GAA GAA AAT GTT TCA GAC GGT TCC CCA AAT GCC GGT TCT GTG GAG CAG   1104
Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu Gln
        355                 360                 365

ACG CCC AAG AAG CCT GGC CTC AGA AGA CGT CAA ACG TAA               1143
Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 380 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Asp Pro Ala Ala Gly
  1               5                  10                  15

Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala Arg
            20                  25                  30
```

Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Gly Ala Leu Pro
35 40 45

Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met Met
50 55 60

Gly Ser Ala Arg Val Ala Glu Leu Leu Leu Leu His Gly Ala Glu Pro
65 70 75 80

Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala Ala
85 90 95

Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly Ala
100 105 110

Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala
115 120 125

Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Ala
130 135 140

Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly
145 150 155 160

Pro Ser Asp Ile Pro Asp Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
165 170 175

Gly Gly Gly Gly Ser Val Glu Ser Asn Val Arg Val Ser Asn Gly Arg
180 185 190

Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys Pro
195 200 205

Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu Thr
210 215 220

Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln Arg
225 230 235 240

Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys Tyr
245 250 255

Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg
260 265 270

Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu Ser
275 280 285

Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro
290 295 300

Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro Ser
305 310 315 320

```
Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg
                325                 330                 335

Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr
            340                 345                 350

Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu Gln
        355                 360                 365

Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1098 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1095

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATG GGA TAC CCT TAT GAT GTG CCA GAT TAT GCC GAT CCG GCG GCG GGG      48
Met Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Asp Pro Ala Ala Gly
  1               5                  10                  15

AGC AGC ATG GAG CCT TCG GCT GAC TGG CTG GCC ACG GCC GCG GCC CGG      96
Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala Arg
            20                  25                  30

GGT CGG GTA GAG GAG GTG CGG GCG CTG CTG GAG GCG GGG GCG CTG CCC     144
Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Gly Ala Leu Pro
        35                  40                  45

AAC GCA CCG AAT AGT TAC GGT CGG AGG CCG ATC CAG GTC ATG ATG ATG     192
Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met Met
    50                  55                  60

GGC AGC GCC CGA GTG GCG GAG CTG CTG CTG CTC CAC GGC GCG GAG CCC     240
Gly Ser Ala Arg Val Ala Glu Leu Leu Leu Leu His Gly Ala Glu Pro
65                  70                  75                  80

AAC TGC GCC GAC CCC GCC ACT CTC ACC CGA CCC GTG CAC GAC GCT GCC     288
Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala Ala
                85                  90                  95

CGG GAG GGC TTC CTG GAC ACG CTG GTG GTG CTG CAC CGG GCC GGG GCG     336
```

```
Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly Ala
            100                 105                 110

CGG CTG GAC GTG CGC GAT GCC TGG GGC CGT CTG CCC GTG GAC CTG GCT  384
Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala
        115                 120                 125

GAG GAG CTG GGC CAT CGC GAT GTC GCA CGG TAC CTG CGC GCG GCT GCG  432
Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Ala
    130                 135                 140

GGG GGC ACC AGA GGC AGT AAC CAT GCC CGC ATA GAT GCC GCG GAA GGT  480
Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly
145                 150                 155                 160

CCC TCA GAC ATC CCC GAT GTC GAG TCA AAC GTG CGA GTG TCT AAC GGG  528
Pro Ser Asp Ile Pro Asp Val Glu Ser Asn Val Arg Val Ser Asn Gly
                165                 170                 175

CGC CCT AGC CTG GAG CGG ATG GAC GCC AGG CAG GCG GAG CAC CCC AAG  576
Arg Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys
            180                 185                 190

CCC TCG GCC TGC AGG AAC CTC TTC GGC CCG GTG GAC CAC GAA GAG TTA  624
Pro Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu
        195                 200                 205

ACC CGG GAC TTG GAG AAG CAC TGC AGA GAC ATG GAA GAG GCG AGC CAG  672
Thr Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln
    210                 215                 220

CGC AAG TGG AAT TTC GAT TTT CAG AAT CAC AAA CCC CTA GAG GGC AAG  720
Arg Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys
225                 230                 235                 240

TAC GAG TGG CAA GAG GTG GAG AAG GGC AGC TTG CCC GAG TTC TAC TAC  768
Tyr Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr
                245                 250                 255

AGA CCC CCG CGG CCC CCC AAA GGT GCC TGC AAG GTG CCG GCG CAG GAG  816
Arg Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu
            260                 265                 270

AGC CAG GAT GTC AGC GGG AGC CGC CCG GCG GCG CCT TTA ATT GGG GCT  864
Ser Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala
        275                 280                 285

CCG GCT AAC TCT GAG GAC ACG CAT TTG GTG GAC CCA AAG ACT GAT CCG  912
Pro Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro
    290                 295                 300

TCG GAC AGC CAG ACG GGG TTA GCG GAG CAA TGC GCA GGA ATA AGG AAG  960
Ser Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys
305                 310                 315                 320
```

```
CGA CCT GCA ACC GAC GAT TCT TCT ACT CAA AAC AAA AGA GCC AAC AGA   1008
Arg Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg
                325                 330                 335

ACA GAA GAA AAT GTT TCA GAC GGT TCC CCA AAT GCC GGT TCT GTG GAG   1056
Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu
            340                 345                 350

CAG ACG CCC AAG AAG CCT GGC CTC AGA AGA CGT CAA ACG TAA           1098
Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
        355                 360                 365
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 365 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Asp Pro Ala Ala Gly
 1               5                  10                  15

Ser Ser Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala Arg
            20                  25                  30

Gly Arg Val Glu Glu Val Arg Ala Leu Leu Glu Ala Gly Ala Leu Pro
        35                  40                  45

Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met Met
    50                  55                  60

Gly Ser Ala Arg Val Ala Glu Leu Leu Leu Leu His Gly Ala Glu Pro
65                  70                  75                  80

Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala Ala
                85                  90                  95

Arg Glu Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly Ala
            100                 105                 110

Arg Leu Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala
        115                 120                 125

Glu Glu Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Ala
    130                 135                 140

Gly Gly Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly
145                 150                 155                 160

Pro Ser Asp Ile Pro Asp Val Glu Ser Asn Val Arg Val Ser Asn Gly
```

```
                                165                 170                 175

Arg Pro Ser Leu Glu Arg Met Asp Ala Arg Gln Ala Glu His Pro Lys
            180                 185                 190

Pro Ser Ala Cys Arg Asn Leu Phe Gly Pro Val Asp His Glu Glu Leu
        195                 200                 205

Thr Arg Asp Leu Glu Lys His Cys Arg Asp Met Glu Glu Ala Ser Gln
    210                 215                 220

Arg Lys Trp Asn Phe Asp Phe Gln Asn His Lys Pro Leu Glu Gly Lys
225                 230                 235                 240

Tyr Glu Trp Gln Glu Val Glu Lys Gly Ser Leu Pro Glu Phe Tyr Tyr
                245                 250                 255

Arg Pro Pro Arg Pro Pro Lys Gly Ala Cys Lys Val Pro Ala Gln Glu
            260                 265                 270

Ser Gln Asp Val Ser Gly Ser Arg Pro Ala Ala Pro Leu Ile Gly Ala
        275                 280                 285

Pro Ala Asn Ser Glu Asp Thr His Leu Val Asp Pro Lys Thr Asp Pro
    290                 295                 300

Ser Asp Ser Gln Thr Gly Leu Ala Glu Gln Cys Ala Gly Ile Arg Lys
305                 310                 315                 320

Arg Pro Ala Thr Asp Asp Ser Ser Thr Gln Asn Lys Arg Ala Asn Arg
                325                 330                 335

Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly Ser Val Glu
            340                 345                 350

Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
        355                 360                 365
```

We Claim:

1. A nucleic acid comprising a nucleotide sequence encoding a chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases.

2. The nucleic acid of claim 1, 14, 22, 23, 26, 27, 28 or 29, which chimeric polypeptide is a fusion protein.

3. The nucleic acid of claim 1, 14, 22, 23, 27, 28 or 29, wherein at least one of the CDK-binding motifs is a CDK-binding motif of a CDK inhibitor protein.

4. The nucleic acid of claim 3, wherein the CDK inhibitor protein is an INK4 protein.

5. The nucleic acid of claim 4, wherein the INK4 protein is selected from the group consisting of p15, p16, p18 and p19.

6. The nucleic acid of claim 3, wherein the CDK inhibitor protein is a CIP protein.

7. The nucleic acid of claim 6, wherein the CIP protein is selected from the group consisting of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$.

8. The nucleic acid of claim 1, wherein at least one of the CDK-binding motifs comprises tandemly arranged ankyrin-like sequences.

9. The nucleic acid of claim 1, 14, 22, 23, 27, 28 or 29, wherein at least one of the CDK-binding motifs comprises a p21/p27 inhibitory domain.

10. The nucleic acid of claim 1, 14, 22, 23, 27, 28 or 29, which chimeric polypeptide comprises a first CDK-binding motif and a second CDK-binding motif, the first and second CDK-binding motifs having different binding specificities, relative to one and other, for cyclin dependent kinases .

11. The nucleic acid of claim 1, which nucleic acid further comprises a transcriptional regulatory sequence operably linked to, and able to control transcription of the nucleotide sequence encoding the chimeric polypeptide.

12. The nucleic acid of claim 1, 14, 22, 23, 27, 28 or 29, wherein the chimeric polypeptide comprises a CDK-binding motif of p16, and a CDK-binding motif of $p27^{kip1}$.

13. The nucleic acid of claim 12, which nucleic acid comprises the coding sequence designated in one of SEQ ID No. 1, 4 or 6.

14. A recombinant transfection system, comprising

(i) a gene construct including a nucleic acid encoding a chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases, and operably linked to a transcriptional regulatory sequence for causing expression of the chimeric polypeptide in eukaryotic cells, and

(ii) a gene delivery composition for delivering the gene construct to a cell and causing the cell to be transfected with the gene construct.

15. The recombinant transfection system of claim 14, wherein the gene construct comprises a viral vector.

16. The recombinant transfection system of claim 15, wherein the viral vector is an adenoviral vector.

17. The recombinant transfection system of claim 15, wherein the viral vector is an adeno-associated viral vector.

18. The recombinant transfection system of claim 15, wherein the viral vector is a retroviral vector.

19. The recombinant transfection system of claim 14, wherein the gene delivery composition comprises a recombinant viral particle.

20. The recombinant transfection system of claim 14, wherein the gene delivery composition is selected from the group consisting of a liposome and a poly-cationic nucleic acid binding agent.

21. The recombinant transfection system of claim 14, wherein the gene delivery composition further comprises a pharmaceutically acceptable carrier for adminstration to an animal.

22. A nucleic acid comprising a nucleotide sequence encoding a chimeric polypeptide comprising a first CDK-binding motif comprising a polypeptide sequence corresponding to tandemly arranged ankyrin-like sequences, and a second CDK-binding motif comprising a polypeptide sequence corresponding to a p21/p27 inhibitory domain.

23. A nucleic acid comprising a nucleotide sequence encoding a chimeric polypeptide comprising (i) a polypeptide sequence corresponding to a CDK-binding motif of an INK4 protein, and (ii) an polypeptide sequence corresponding to a CDK-binding motif of a CIP protein.

24. The nucleic acid of claim 23, wherein the INK4 protein is selected from a group consisting of p15, p16, p18 and p19.

25. The nucleic acid of claim 23, wherein the CIP protein is selected from the group consisting of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$.

26. A nucleic acid comprising a nucleotide sequence encoding a chimeric polypeptide comprising (i) a CDK-binding motif of p16 or p15, and (ii) a p21/p27 inhibitory domain of $p21^{CIP1}$, $p27^{KIP1}$ or $p57^{KIP2}$.

27. A viral vector comprising a nucleotide sequence encoding a chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases, which viral vector is capable of infecting mammalian cells and expressing the chimeric polypeptide.

28. An adenoviral vector comprising a nucleotide sequence encoding a chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases.

29. A chimeric polypeptide comprising CDK-binding motifs from two or more different proteins which bind to cyclin dependent kinases.

30. The chimeric polypeptide of claim 29, which chimeric polypeptide is a fusion protein.

31. The chimeric polypeptide of claim 29, wherein at least one of the CDK-binding motifs is a CDK-binding motif of a CDK inhibitor protein.

32. The chimeric polypeptide of claim 31, wherein the CDK inhibitor protein is an INK4 protein.

33. The chimeric polypeptide of claim 32, wherein the INK4 protein chimeric is selected from the group consisting of p15, p16, p18 and p19.

34. The chimeric polypeptide of claim 31, wherein the CDK inhibitor protein is a CIP protein.

35. The chimeric polypeptide of claim 34, wherein the CIP protein chimeric is selected from the group consisting of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$.

36. The chimeric polypeptide of claim 29, wherein at least one of the CDK-binding motifs comprises tandemly arranged ankyrin-like sequences.

37. The chimeric polypeptide of claim 29, wherein at least one of the CDK-binding motifs comprises a p21/p27 inhibitory domain.

38. The chimeric polypeptide of claim 31, which chimeric polypeptide comprises a first CDK-binding motif and a second CDK-binding motif, the first and second CDK-binding motifs having different specificity for CDK binding relative to one and other.

39. The chimeric polypeptide of claim 29, which chimeric polypeptide comprises a CDK-binding motif of p16, and a CDK-binding motif of $p27^{kip1}$.

40. The chimeric polypeptide of claim 39, which chimeric polypeptide comprises the fusion sequence designated in SEQ ID No. 2, 5 or 7.

41. The chimeric polypeptide of claim 29, formulated in pharmaceutically acceptable carrier for delivery to a mammal.

42. The chimeric polypeptide of claim 41, wherein the pharmaceutically acceptable carrier includes a liposomes.

43. A transgenic animal comprising cells which harbor a nucleic acid of claim 1.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 97/00569

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C12N15/62 C12N15/86 C12N15/87
A61K38/17 A01K67/027 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, (1995 MAY 11) 375 (6527) 159-61., XP002031641 LUO, Y. ET AL.: "Cell - cycle inhibition by independent CDK and PCNA binding domains in p21Cip1."	1-3,6,7, 9,11, 29-31, 34,35,37
Y	see the whole document	14-21, 27,41-43

Y	WO 95 28483 A (COLD SPRING HARBOR LAB) 26 October 1995 see page 24, line 27 - page 25, line 33 see page 36, line 14 - page 46, line 10	14-21, 27,41-43

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1997	03.06.1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

Intern. ..al Application No
PCT/US 97/00569

C.(Continuation) DOCUMENTS C NSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESSION, vol. 5, no. 2, 1995, pages 127-156, XP000572011 MACLACHLAN, T. ET AL.: "CYCLINS, CYCLIN-DEPENDENT KINASES AND CDK INHIBITORS: IMPLICATIONS IN CELL CYCLE CONTROL AND CANCER" see page 137, right-hand column, paragraph 2	1-3,6,7, 29-31, 34,35,37
A	see page 137 - page 141	
A	MOLECULAR BIOLOGY OF THE CELL, vol. 15, no. 5, May 1995, pages 2672-2681, XP000614380 HIRAI, H. ET AL.: "NOVEL INK4 PROTEINS, P19 AND P18, ARE SPECIFIC INHIBITORS OF THE CYCLIN D-DEPENDENT KINASES CDK4 AND CDK6" cited in the application see the whole document	4,5,8, 24,33,36
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 15, February 1995, pages 997-1004, XP000673881 OGAWA, N. ET AL.: "Functional domains of Pho81p, an inhibitor of Pho85p protein kinase, in the transduction pathway of Pi signals in Saccharomyces cerevisiae" cited in the application	
A	NATURE, vol. 374, 23 March 1995, pages 386-388, XP000608229 CHEN, J. ET AL.: "SEPARATE DOMAINS OF P21 INVOLVED IN THE INHIBITION OF CDK KINASE AND PCNA"	

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INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inte. .onal Application No
PCT/US 97/00569

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2186639 A	15-10-95
		EP 0755445 A	29-01-97